In-situ Biofilm Formation in Hyper Alkaline Environments

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Abstract

Lime manufacture in the UK has resulted in the generation of a number of alkaline sites (>pH 11.0) with complex indigenous microbial populations. Within the present study, retrievable cotton samples were used to investigate the fate of cellulose, the primary carbon source, within three sites aged from ≈25 to 140 years. Following three months incubation in situ, biofilms had formed on all cotton samples in these extreme pH conditions; with matrices comprised of carbohydrates, proteins, lipids and eDNA. Biofilms from the older sites contained greater amounts of eDNA, a structural component that aids the production of a denser biofilm. The age of the sites correlated with a shift from polysaccharides composed of β 1,4 and β 1,3 linked sugars to those composed of pyranosyl sugars within the older sites. These changes were reflected in the active biofilm communities which shifted from being *Clostridiales* dominated in the youngest site to *Proteobacteria* dominated in the older sites. The study demonstrates that the microbial communities resident in anthropogenic alkaline sites are able to form biofilms at pH values >pH 11.0 and that these biofilms evolve towards *Proteobacteria* dominated communities employing eDNA and pyranosyl sugar based polysaccharides to build the biofilm matrix.

Keywords: Biofilm; Hyperalkaline; Lime manufacture; Cellulose; Isosaccharinic acid; eDNA

Introduction

The UK has a long and rich industrial heritage which has generated a wide range of terrestrial environments which pose significant challenges to their indigenous microbial populations. Amongst these are a range of anthropogenic alkaline environments (Gomes et al., 2016), which in the UK are commonly linked to the waste disposal practices of historic lime manufacture (Milodowski et al., 2013). Lime manufacture in the UK dates back to the Roman period (Williams, 2004) with a range of historical sites in northern England originating from the 17th to the 20th century (Johnson, 2008, White, 2006, Milodowski et al., 2013).

One such site in Derbyshire, UK has waste disposals dating back ca. 140 years (Milodowski et al., 2013) which have generated an alkaline plume where pH values of greater than pH 12.0 are common. Despite this harsh geochemical environment, the site supports an extensive and diverse bacterial (Williamson et al., 2013, Bassil et al., 2014, Burke et al., 2012) and archaeal (methanogenic) populations (Rout et al., 2015, Charles et al., 2015) capable of a wide range of metabolic and energy generating processes (Bassil et al., 2014, Rout et al., 2015). The alkaline conditions generated by these wastes result in the *in-situ* chemical hydrolysis (Knill and Kennedy, 2003) of cellulosic materials, i.e. plant matter, demonstrated by the detection of iso-saccharinic acids (ISA) in these sediments (Rout et al., 2015).

Biofilm formation is recognized as a key microbial adaption strategy against extreme environmental conditions (Davey and O'toole, 2000), and biofilm matrix materials have been shown to provide protection against extreme pH values (Charles et al., 2017). The work reported here investigates the ability of microbial populations in three temporal and geographically distinct lime kiln waste sites to colonize and form cellulose associated biofilms at pH values >pH 11.0. These sites have been subject to lime contamination from ≈25 to 145

years allowing for a comparative analysis of the biofilm forming capabilities of the indigenous microbial populations.

Methods

Site Descriptions

Three geographically distinct UK lime kiln waste sites were chosen for this investigation. The first sites is the previously described Buxton site (Site B, Figure 1A) which began operation in 1872 and operated up until 1944 (Milodowski et al., 2013). The other two sites are located in North Yorkshire, Site H has a hyper alkaline lagoon formed when lime kiln wastes were disposed of in the 1940's (Figure 1B). The third site (Site T) is also adjacent to a limestone quarry and features a drainage basin which manages an alkaline leachate generated by rain water percolation through historical waste deposits. All three sites have an interface area where the alkaline waters inundate a vegetated marginal area (Figure 1C).

[Figure 1 near here]

Site Investigation

At the interface between the alkaline leachate and the surrounding land a series of 2.2 cm Ø boreholes were emplaced to an approximate depth of 0.5 m at all three sites. These boreholes were encased with plastic liners that were perforated for the bottom ≈5 cm. Nylon mesh bags containing approximately 1 g of sterile, de-waxed, cotton (Charles et al., 2015) were then placed at the bottom of each borehole. A total of 6 boreholes were placed into Site H and 5 boreholes into Site T. For Site B, the 4 boreholes previously described by Charles et al. (2015) and Rout et al. (2015) were used. Pore water and sediment samples were collected prior to the emplacement of the cotton and were analyzed for a range of parameters as previously described in Rout et al. (2015).The pH of the pore water was recorded *in-situ* before and after

emplacement via a handheld portable pH meter with calibrated electrodes (Mettler Toledo, UK).

After 3 months the cotton and samples of the local pore waters were collected and sealed in airtight containers along with anaerobic gas packs (Anaerogen, Oxoid, UK) for transport. Pore waters were then analyzed for VFA and ISA content and small portions of the cotton were used for ISA content determination. VFA and ISA concentrations were analyzed via gas and anion exclusion chromatography as described by Rout et al. (2015). Cotton not for immediate use was fixed overnight using 4% paraformaldehyde in phosphate-buffered saline (PBS) and stored at -20°C in a TRIS-HCl ethanol based solution described in Charles et al. (2015).

Microscopy

The EPS composition and biofilm morphology was determined via confocal laser scanning microscopy (CLSM) at the Bio imaging center of Leeds University using a Zeiss LSM880 inverted confocal microscope with image analysis performed using Zen 2.1 (Zeiss Microscopy). Small sections of fixed cotton were stained using the following compounds in accordance with methods outlined in Chen et al. (2007): Calcofluor white for the visualization of β-1,4 and β-1,3 polysaccharides (Sigma, UK), Nile red (Fisher, UK) for lipids and hydrophobic sites, Concanavalin A, Tetramethylrhodamine Conjugate (Fisher, UK) for α-Mannopyranosyl, α-glucopyranosyl sugars, FITc (Fisher, UK) for protein and Syto 63 (Fisher, UK) for total cells and extracellular DNA. Biofilms were further investigated using scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX) via a Quanta FEG 250 scanning electron microscope. Fixed samples were dehydrated using a serial ethanol dilution of 25, 50, 75 and 100% for 2 minutes per step and sputter coated via a gold palladium plasma (CA7625 Polaron, Quorum Technologies Ltd, UK) before visualization.

RNA Extraction and Community Analysis

In order to focus on the active microbial population and to avoid potential contamination from bacterial spores, 16S rRNA, rather than 16S rDNA community analysis was carried out. Cotton samples were washed with pH 4 and then pH 7 PBS under an inert nitrogen atmosphere to remove any transient microorganisms and to neutralize the cotton samples. Samples were then cut into small sections using sterile scissors and placed in a 15ml Falcon tube with metal beads provided in the RNA Powersoil kit (Mo-BIO, Carlsbad). DNA and RNA was then co-extracted using methods outlined in Griffiths et al. (2000). Briefly; 2.5ml phenol-chloroform-isoamyl alcohol (25:24:1) (pH 8.0) (Sigma, UK) and 2.5ml CTAB extraction buffer with 0.5ml β-marcaptoethanol (Fisher, UK) were added to the bead tube and beaten for 20 minutes. Samples were centrifuged and the aqueous phase extracted and mixed with an equal volume of chloroform-isoamyl alcohol (24:1) (Sigma, UK). Samples were centrifuged again and the aqueous phase was extracted and the DNA/RNA precipitated using ethanol. RNA was isolated by digesting the extracted nucleic acid mix via the DNase 1 kit (Sigma-Aldrich, UK) and purifying the RNA using an RNeasy mini elute clean up kit (Qiagen, UK). After elution RNA from related samples was pooled to increase the yield and concentrated using ethanol precipitation. cDNA was generated from the pooled RNA for each site using a Tetro cDNA synthesis kit with random hexamer primers (Bioline, UK). The V4 region of the 16S rRNA gene was amplified using duel primers 519F (5'CAGCMGCCGCGGTAA'3) and 785R (5'TACNVGGGTATCTAATCC'3) (Klindworth et al., 2013, Jung et al., 2011) with the following overhangs 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG'3 and 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG'3, respectively. PCR products were purified using a Qiaquick PCR purification kit (Qiagen, UK) and 16S microbial Community analysis was carried out via a MiSeq platform (Illumina, USA) at 250bp paired ends with chimera detection and removal performed via the UNCHIME algorithm in the MOTHUR suite

(Schloss et al., 2009) (Chunlab, South Korea). Assignment of OTU's was performed using a CD-HIT clustering method with a 95% cut-off value and specific OTU analysis was carried using Taxon XOR analysis in the CLcommunity software suite (Chunlab, South Korea) with taxonomic assignment performed against the EZtaxon database (Kim et al., 2012).

Accession numbers

16s rRNA sequence data was uploaded to the NCBI sequence read archive under the accession number SRP073428.

Results

In all three cases the pore water pH associated with the cotton samples, post incubation was ≥ pH 11.9 (Table 1). The alkaline nature of the environments was confirmed by the presence of both the alpha and beta stereoisomers of ISA; compounds only generated by the alkaline hydrolysis of cellulose under anoxic conditions. This ISA was accompanied at all sites by acetic acid indicating an actively fermenting microbial populations.

[Table 1 near here]

Microbial consortia colonized the emplaced cotton at all three sites with the associated biofilms punctuated with mineral deposits predominantly composed of carbon, calcium and oxygen (Figure S1) as would be expected in a calcium dominated environment. The colonization of the cotton showed a progression with the youngest site (H) having shallower (13μm) less dense biofilms, these increased in depth in site H (28μm), and both depth and density at site B (23μm) (Figure 2). All three biofilms possessed complex matrix components comprised of proteins, carbohydrates, cells, extracellular DNA (eDNA) and lipids (Figure 2), however there were key differences in the matrix compositions. For example, eDNA was less abundant at site T, concentrated at the cotton surface in Site B and present throughout the biofilm at site B. Pyranosyl and β 1,4 and 1,3 linked polysaccharides were present at all sites, however the site

B biofilm was dominated by pyranosyl sugars whilst the site T biofilm relied more heavily upon β 1,4 and 1,3 linked residues with site H having both polysaccharides in equal abundance. This reflects the position of Site H as an intermediate between the older and younger sites.

[Figure 2 near here]

In line with the differences in the biofilm matrix components, analysis of the active microbial communities at each site, indicated that the Site B and Site H consortia were more similar to each other than to the Site T consortia (Figure 3). Reads associated with the *Protobacteria* comprised the greatest proportion of both the Site B (62.0%) and Site H (67.4%) communities, compared to 20.1% of the Site T community. Despite this similarity, within the site B community, 29.4% of the community was comprised of taxa of the *Burkholderiales*, with a further 20.6% being within the *Rhizobiales*. Despite these two Orders being present within the Site H community, comprising 11.2% and 9.4% respectively, taxa of the *Enterobacteriales* (16.2%) and the *Pseudomonadales* (23.4%) represented the dominant *Proteobacteria* of this community. Taxa of the *Enterobacteriales* were the most prevalent *Proteobacteria* within the Site T community (8.7%).

[Figure 3 near here]

Taxa of the *Firmicutes* were the most prevalent of the Site T community, with taxa of the *Clostridiales* comprising 35.8% of the community. *Firmicutes* were also present within the Site B (7.9%) and Site H (10.1%) communities. Taxa of the *Actinobacteria* were present in all three communities, but comprised a much larger component of the Site H community (14.0%), within this phylum taxa were identified as being from the *Corynebacteriales* (5.6%) and *Micrococcales* (6.8%). In a similar manner, taxa associated with the *Acidobacteria* were more prevalent in the Site B community (13.0%) than that of Site T (7.6%) or Site H (1.4%) communities. Within the Site B community, 10.4% of the total reads were associated with the

Order designation EU686603. *Planctomycetales* (4.8%) and *Verrumicrobiales* (3.0%) were also more prevalent in the site B community than in the biofilms of the other two sites. The biofilm community of Site T comprised a number of taxa of the *Bacteroidales* (5.4%), AF544207 (2.1%), FR720650 (2.3%) and *Fibrobacteriales* (3.3%), which were all in a greater abundance than those of the other two sites.

Discussion

Our understanding of anthropogenic alkaline environments has gained traction in recent years (Burke et al., 2012, Milodowski et al., 2013) primarily driven by their potential as analogues for aspects radioactive waste disposal practices (Rout et al., 2015). However, the harsh geochemical environments these sites create provide an insight into the ability of microbial life to flourish at environmental extremes.

Within this present study we have identified the biofilm forming potential of the micro-organisms from three of these alkaline environments varying in age from 25-140 years. The biofilms existed on the surface of cotton cellulose emplaced in situ, where the cotton clearly acts as a surface for biofilm formation. The carbon source used by these biofilms is likely to be a combination of the cellulose and the products of its anoxic, alkaline hydrolysis since there was limited evidence of the pits and grooves associated with microbial anaerobic cellulose degradation at circumneutral conditions (McDonald et al., 2012). *Fibrobacter* sp, cellulolytic organisms (Ransom-Jones et al., 2012, McDonald et al., 2012) were detected in all three biofilm communities and ISA could be extracted from the cotton indicating that that they were available as a carbon source for the biofilm communities. In addition recently isolated strictly anaerobic, ISA degrading bacteria also contain cellulosomes which would enable cellulose degradation (Rout et al., 2017). There was, however, no evidence of the acidification observed

in some in-vitro studies (Bassil et al., 2015) indicating that the buffering capacity of all three sites was sufficient to counter act any acidification associated with fermentation processes.

Extracellular DNA was a key matrix component at all three sites and is likely to provide structural support by anchoring the biofilm to the cellulose surfaces (Charles et al., 2017). This stabilizing influence of eDNA is enhanced via thermodynamically favorable interactions with Ca^{2+} ions (Das et al., 2014). Sequestration of Ca^{2+} may also impact on pH buffering by the partial prevention of $CaCO_3$ precipitation (Arp et al., 1999), although the formation of carbonate deposits was observed in all three biofilms. The degree of eDNA present within the biofilm appeared to be less within the youngest site (T), which coincided with fewer *Proteobacteria*; in particular *Burkholderiales* and *Rhizobiales*. The structural role of eDNA in the formation of *Pseudomonas* biofilms has been reported by a number of authors (Wang et al., 2015, Ma et al., 2009), our findings suggest that this may be a characteristic of a number of taxa within this Phylum.

The community of site H also had taxa of the *Enterobacteriales* and *Pseudomonadales* of the *Proteobacteria* which may suggest that the geochemistry, or age of this site is influencing the components of the community. This was further suggested by the high abundance of *Corynebacteriales* within the biofilm represented by *Corynebacterium sp* at the genus level. *Corynebacterium* are largely described based on their lipophilic properties (Kosaric, 2001), which coincides with a diminished lipid content within the site H biofilm compared to that of B and T. The increased lipid content in these biofilms correlated with the increased detection of *Acidobacteria*, however many of the species within this phylum remain uncharacterized (Kielak et al., 2016) and consequently this link remains to be verified. The production of lipids within the biofilm will increase hydrophobicity and reduce wetting (Epstein et al., 2011) which will in turn reduce the impact of the alkaline pore waters. Lipids may also maintain the pH buffering capacity of the biofilm matrix if the acidic phospholipids reported in the membranes

of alkaliphilic bacteria (Enomoto and Koyama, 1999) have been employed in matrix construction.

Carbohydrates were also a key component of all three biofilms, however there was a shift from β1,4 and β 1,3 polysaccharides in the youngest site to pyranosyl sugars in the oldest site. Interestingly the biofilm of site H contained similar amounts of each type. Whether this is a true indicator of the time of exposure to alkaline conditions or a result in the slight differences in community composition is unclear. Proteins were present within all three biofilms. In the younger site biofilms, the proteins were concentrated in bottom 50% of the matrix, where as they were distributed all the way through the matrix from the oldest site (B). Recent findings suggest that proteins are prevalent in both single (Liu et al., 2015) and multiple organisms biofilms (Charles et al., 2015), where they play a number of key roles including the sorption of inorganic and organic ions, enzymatic reactions and protection from environmental conditions (Flemming and Wingender, 2010).

This investigation demonstrates that in situ biofilm formation is not only possible but common despite the extreme alkaline conditions (>pH 11.0) generated by the disposal of lime kiln wastes. The thickness and density of the biofilms observed suggests that the microbial communities in the older sites (>100 years) are better adapted to this harsh environment. Despite the age of the site, eDNA plays a key structural function in all the biofilms formed and its prevalence correlates with the presence of taxa of the *Burkholderiales, Rhizobiales*, *Enterobacteriales* and *Pseudomonadales* and a reduction in *Clostridiales*. This suggests that the presence of these taxa is essential for the formation of stable biofilms in these geochemically aggressive environments.

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Disclosure statement

There is no conflict of interest.

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Site	Approximate Age	pH	µM	(mg (g dry wt)$^{-1}$	
			Acetic Acid	α-ISA	β-ISA
B	140	11.9*	208.9*	2.34*	0.85*
H	50	13.5-13.6	828.0±757.3	1.06 ± 0.42	1.15 ± 0.47
T	25	12.4-13.1	313.7±202.6	1.69 ± 0.12	1.43 ± 0.17

*Previously reported in Charles et al (2015). Mean±SE

Table 1: pH and ISA content of the porewaters associated with the cotton samples.

Figure 1: Terrain of site B (A), Site H (B) and Site T (C).

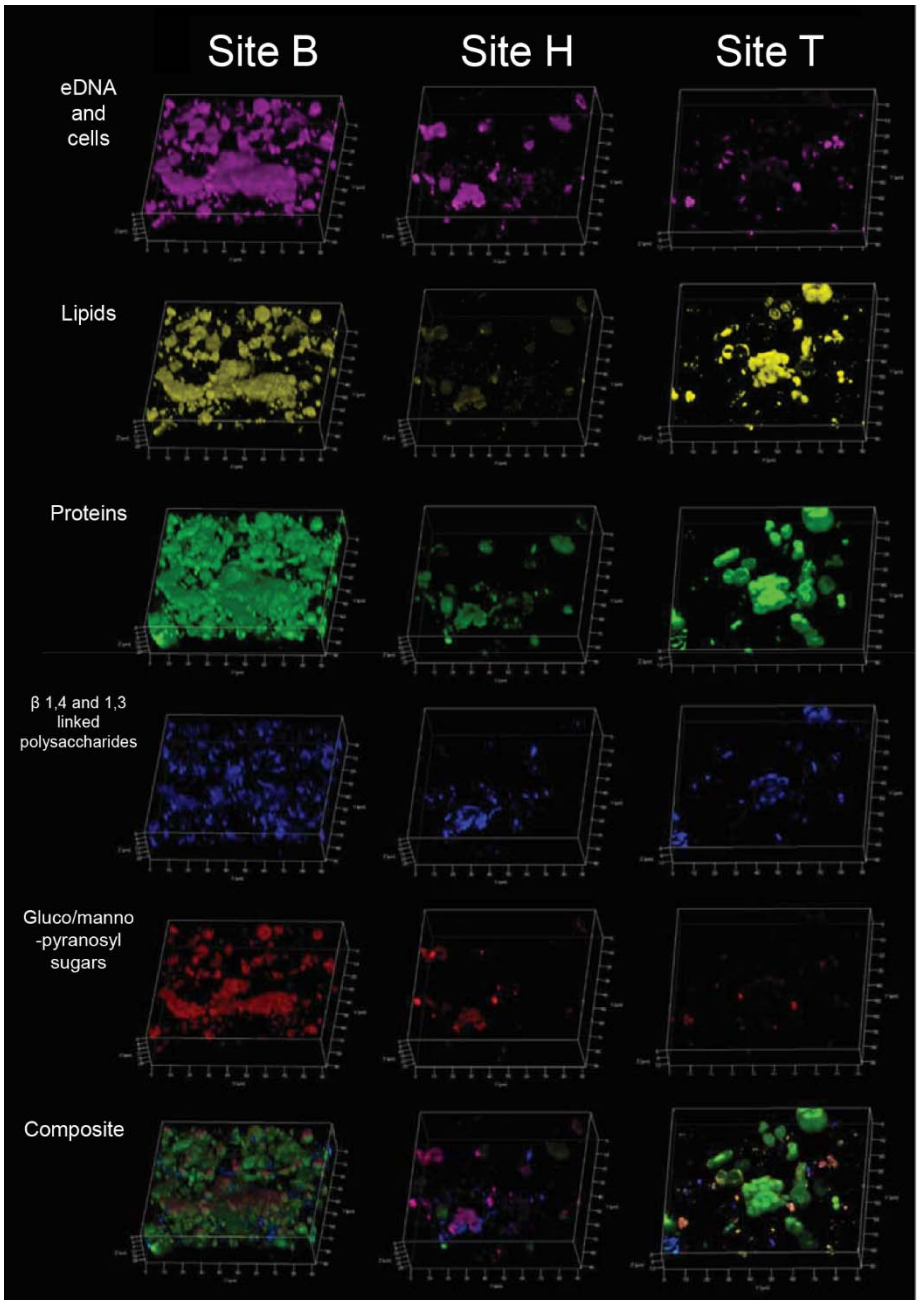

Figure 2: Biofilms from all three sites were composed or a range of complex polymeric components. CLSM imaging showed that the biofilms were a complex mixture of proteins, carbohydrates, cells, extracellular DNA and lipids.

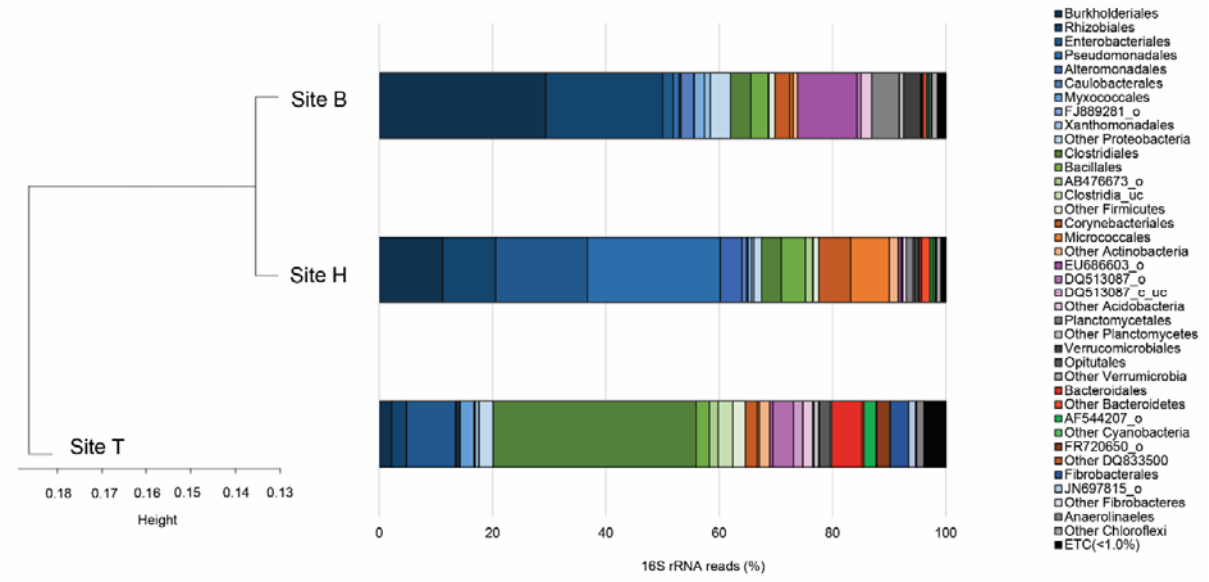

Figure 3: Community analyses of the three biofilm communities indicated that the site B and site H communities showed greatest homology, dominated by taxanomic Orders of the *Proteobacteria* Phylum. Although also comprising these Orders, the Site T community had a greater abundance of *Clostridiales* of the *Firmicutes* phylum.